[Cancer Biology & Therapy 6:7, e1-e8, EPUB Ahead of Print: http://www.landesbioscience.com/journals/cbt/abstract.php?id=4327; July 2007]; ©2007 Landes Bioscience

Research Paper

Silencing of *Lactotransferrin* Expression by Methylation in Prostate Cancer Progression

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Original manuscript submitted: 02/01/07
Manuscript accepted: 04/20/07

This manuscript has been published online, prior to printing for Cancer Biology & Therapy, Volume 6, Issue 7. Definitive page numbers have not been assigned. The current citation is: Cancer Biol Ther 2007; 6(7):
http://www.landesbioscience.com/journals/cbt/abstract.php?id=4327
Once the issue is complete and page numbers have been assigned, the citation will change accordingly.

KEY WORDS

LTF, downregulation, methylation, PSA doubling time, chemoprevention

ABSTRACT

Background: Cancer cells gain selection advantages by the coordinated silencing of protective and by the activation of cell proliferation/cell survival genes. Evaluations of epithelial cell transcriptome of benign and malignant prostate glands by laser capture microdissection (LCM) identified Lactotransferrin (LTF) as the most significantly downregulated gene in prostate cancer (CaP) cells ($p < 10–6$). Frequent downregulation, significant association of LTF with PSA recurrence-free survival in CaP patients and the established anti-tumorigenic effects of LTF in experimental cancer models have provided impetus to evaluate LTF expression features and mechanisms in CaP specimens.

Methods: LTF mRNA expression analysis was performed in LCM derived benign and malignant prostate epithelial cells by using Affymetrix GeneChip and QRT-PCR. LTF protein expression was assessed in tissue specimens by immunohistochemistry and in serum samples from CaP patients compared to healthy male control by using ELISA. Mechanism of LTF downregulation was analyzed in 5-azadeoxycytidine treated LNCaP and LAPC4 cells using MALDI-TOF MS. Proliferation and cell cycle analysis of CaP cells by FACS flow cytometry was assessed in LNCaP cell cultures.

Results: Quantitative analysis of LTF mRNA expression in tumor cells revealed marked downregulation of LTF with significant associations to decreased PSA recurrence-free survival of CaP patients ($n = 100$, $p \leq 0.0322$). Moreover, low levels of LTF protein expression was observed in tumor tissues as well as in sera from CaP patients ($p \leq 0.0001$). LTF promoter downstream CpG island methylation was found in LNCaP and LAPC4 cells. Furthermore, replenishing of LTF by supplementing growth media with LTF protein resulted in the reduced cell growth. Cell cycle analysis revealed robust increases in apoptosis in response to LTF treatment.

Conclusion: This study highlights the potential for LTF in chemoprevention and to become a biologically relevant prognostic marker of CaP, suggesting that silencing of the LTF gene may be causally linked to CaP progression.

INTRODUCTION

While recent trends in stabilizing incidence and decreasing mortality rates are encouraging, prostate cancer (CaP) remains a major health burden for American men.[1] Similar to other cancers, prostate cancer develops through a multi-step process of genetic changes, each providing some type of growth advantage for cells, leading to their progressive conversion into aggressive cancer cells. Intensive investigations on CaP-specific genetic alterations are beginning to define common genetic changes in CaP.[2-4] Chromosomal rearrangements/translocations leading to activation of ETS transcription factors (ERG, ETV1 and ETV4) or methylation mediated silencing of protective genes such as GSTP1 appear to be most common potentially causal gene alterations in CaP.[2,5] Expression alterations of the GSTP[1,6] DD[3,7] AMACR,[8] and proto-oncogene, ERG,[9] represent the most frequent CaP associated gene expression changes.

LTF, a non-heme iron binding glycoprotein, belongs to the transferrin gene family and arose from an ancient intragenic duplication.[10] LTF was first discovered in milk and was also found in a variety of secretions derived from glandular epithelium cells, such as prostate and salivary glands as well as in many biological secretions including tears and semen. LTF plays an immunomodulatory role and participates in inflammatory response. A number of reports suggested that LTF has an antitumorigenic role[11] utilizing a variety of mechanisms including regulation of NK (natural Killer) cell activity,[12] modulation of expression of G_1 proteins,[13] and enhancement of apoptosis.[14] LTF has anti-tumor function and could inhibit development of tumors in animal models.[10,15]

Bovine lactotransferrin (bLTF) has been found to significantly inhibit colon, esophagus, lung, bladder and liver cancers in rats.[16-20] Moreover, bLTF was shown to be chemopreventive in liver carcinogenesis induced by diethyl nitrosamine (DEN) alone or by DEN plus 2-amino-3, 8-dimethylimidazo[4,5-f]quinoxaline (MeIQx), or in MeIQx-induced colon carcinogenesis in rat animal models.[21]

However, the role of LTF in human prostate cancer remains to be defined. In this study, we evaluated the expression of LTF gene in CaP specimens and assessed the prognostic value of decreased or lost LTF expression in CaP progression. Furthermore, we investigated the mechanism of LTF downregulation and the cell growth inhibitory effects of LTF in prostate cancer cell model systems.

MATERIALS AND METHODS

GeneChip® analyses. Total RNA was isolated from LCM derived tumor and benign epithelial cells according to protocol number WRAMC WU# 04-2871-98k. The RNA was quantitated, amplified, biotinylated, and hybridized to high-density oligonucleotide human genome array HG U133A (Affymetrix, Santa Clara, CA).[9] The gene expression data from paired benign and malignant prostate epithelial cells from 20 patients (40 GeneChips®) were subjected to multidimensional scaling (MDS) analysis as described previously.[9]

Validation of LTF expression by QRT-PCR. Total RNA isolated from the LCM derived tumor and benign epithelial cells, was converted to cDNA (Sensiscript, Qiagen, Valencia, CA). Quantitative gene expression analysis was performed by TaqMan-based QRT-PCR as described.[9] LTF copy number was determined in tumor and benign samples (n = 100) and normalized to GAPDH copy numbers.[22] Statistical analyses were performed using the SAS software package (version 9.0, SAS Institute Inc., Cary, NC). PSA recurrence free survival was correlated with LTF expression ratio.[23]

Northern blot analysis. Twenty micrograms of total RNA from LNCaP and LAPC4 cells and RNA Millennium™ Markers (Ambion Inc., TX, USA) were electrophoresed in denaturing formaldehyde 1% agarose gels. Following overnight capillary transfer to Protran Pure Nitrocellulose Transfer and Immobilization Membranes (Schleicher & Schuell Keene, N.H., USA). Blots were probed with LTF cDNA of 2.9 Kb in size (OriGene Technologies, Inc., Rockville, MD USA), GAPDH-Mouse DECAprobe template and Millennium Marker™ Probe Template (Ambion Inc., TX, USA), which were radioactively labeled with α-32p-dCTP (GE Healthcare, Buckinghamshire, UK) using a random-primed DNA labeling kit named, Amersham Rediprime™ II Random Prime Labeling Systems (GE Healthcare, Buckinghamshire, UK). Hybridization was carried out for approximately 18 h in NorthernMax Prehybridization/ Hybridization buffer (Ambion Inc., Tex., and USA). The membrane was washed twice with 1x SSC and 0.1% SDS for 15 min each and then washed twice with 0.25 x SSC and 0.1% SDS for 15 min each and autoradiographed. Sizes of the target genes were determined from RNA Millennium™ Markers on the autoradiograph.

Analysis of LTF protein expression by immunohistochemisrty (IHC). All tissues were fixed in 10% neutral buffered formalin and were paraffin embedded. Prostate tissue specimens were selected with the criteria of having sufficient tissue within the paraffin block to perform the immunohistochemistry (IHC). Tissue sections (100 μm) of the radical prostatectomy derived whole-mount prostate specimens from 30 CaP patients were stained with H&E and anti-LTF by IHC.[24]

Determination of LTF protein concentration in CaP patient Serum. Serum LTF protein concentration of biopsy-verified CaP patients who underwent radical prostatectomy at Walter Reed Army Medical Center (WRAMC) was compared to CaP-free control group with normal digital rectal examination (DRE) and a low serum PSA (<2.5 ng/ml). Serum LTF levels were measured in a cohort of 34 CaP patients and 35 healthy male controls using Bioxytech Lacto-f-EIA (OxisResearch, Portland, OR) and a Multiskan Ascent ELISA plate reader according to the manufacturer's recommendation.

Activation of LTF expression in LNCaP cells by 5-azadeoxycytidine treatment. LNCaP and LAPC4 cells were treated with 5 μM of 5-azadeoxycytidine DNA methyl transferase inhibitor (Sigma-Aldrich Co., St. Louis, MO) for 14 days at concentration of 5 μM with the change of cell culture media every two days (concentration was optimized prior to the actual experiment to avoid toxicity). Quantitative methylation analysis of genomic DNA from treated and untreated LNCaP cells were performed by SequEnom® (San Diego, CA) using EpiTYPER, a bisulfite-treatment-based MALDI-TOF MS method for detection and quantitation of methylation.[25] To amplify the bisulfite modified +200 to +600 CpG-rich region of the LTF gene, a primer pair was designed as follows: forward primer, 5'-GGGGTAAAGTTTTGAATAAAGGGG-3' and reverse primer 5'-TAAAAAACCCAACTATTCCTCC-3'. The GSTP1 gene -300; +100 region was amplified by using the following primer set: forward 5' TGGGAAAGAGGGAAAGGTTTTTT-3' and reverse 5'-CCCATACTAAAAACTCTAAACCCCATC-3'. For gene expression analysis total RNA was extracted from untreated and 5-azadeoxycytidine treated LNCaP and LAPC4 cells using RNeasy Mini kit (QIAGEN®, Valencia, CA). For gene expression analysis total RNA was extracted from untreated and 5-azadeoxycytidine treated LNCaP and LAPC4 cells using RNeasy Mini kit (QIAGEN®, Valencia, CA). The RNA was transferred on nitrocellulose membrane to asses the expression of LTF by Northern Blot analysis in both LNCaP and LAPC4 cells as described above. GAPDH expression was assessed as the quality control of RNA samples.

Effects of LTF on LNCaP cell proliferation and cell cycle. LNCaP cells (5 x 10^4 cells/well, passage 28) in RPMI medium were plated into 12-well plates and allowed to grow in a 5% CO_2 incubator at 37°C for 24 hours. LTF dose response was assayed by supplementing the media with various concentrations of LTF protein (Sigma-Aldrich, St. Louis, MO) from a 1000 μM stock solution to final concentrations of 0, 10, 30 and 50 μM. Cell growth was determined at 24, 48, 72 and 96 hours. The experiment was performed in triplicate (n = 3) and repeated twice. Cell morphology was monitored and photographed using an inverse microscope (DMIRE2, Leica Microsystems, Bannockburn, IL). Statistical analysis was performed with SPSS version 12 software package. $p < 0.05$ values were considered to be statistically significant. For cell cycle analysis Propidium Iodide (PI) staining and FACS analysis was performed. The fluorescent dye Propidium Iodide preferentially binds to double-stranded nucleic acids and allows fluorescent intensity to be used as an indicator of the cellular DNA content. LNCaP cells were grown to ~70% confluence in 25-cm^2 flasks and were treated with 10 μM of human lactoferrin for 24 h and 48 h at 37°C. Following treatments, the adherent cells were trypsinized and washed twice 1x PBS and then fixed with 70% cold ethanol at -20°C for 30 min. Cells were washed twice with cold PBS following ethanol fixation. Cells were resuspended in PBS containing 10 μg/ml RNase (Boehringer Mannheim, Indianapolis, IN) and incubated at 37°C for 30 min to remove double stranded RNA. Subsequently, cells were stained with PI (Boehringer Mannheim, Indianapolis, IN) at a concentration of 50 μg/ml and stored at 4°C in the dark until flow cytometry was

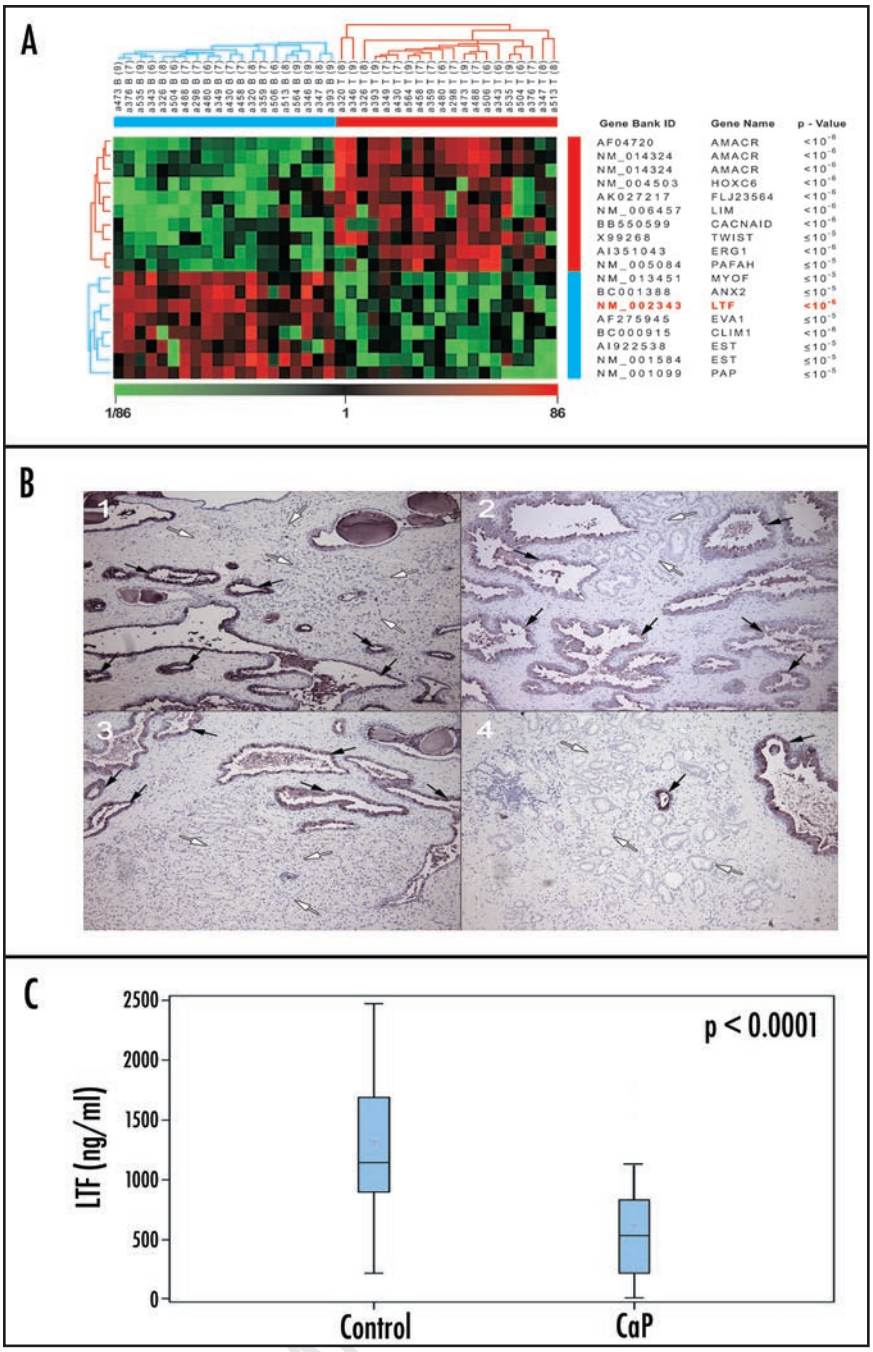

Figure 1. LTF is downregulated in prostate cancer. (A) Gene expression signature representing LTF downregulation in prostate tumor (T) cells compared to matched benign (B) epithelium. The heat map displays tumor and benign expression of LTF in 20 CaP patients (9 with High Risk and 11 with Moderate Risk form of CaP) based on signals obtained by probe set 202018_s_at (Gene Bank ID: NM_002343) on the Affymetrix GeneChip HG U133A. The color scale from 1/86 fold to 86-fold ratio is shown under the heat map. Gleason scores are shown in parenthesis at the top of the heat map. Expression data was analyzed by multidimensional scaling (MDS) using the MATLAB package (http://arrayanalysis.nih.gov/marray.html) from NHGRI. Genes with the most significant expression differences were selected by t-test (by lowest p-Values). (B) LTF immuno-staining of tissue sections (4 μm) of 30 whole -mount prostate specimens revealed overall high levels of LTF protein in benign glands as compared to malignant glands. Panel B shows LTF protein expression in four prostate tissue sections of CaP patients by IHC using anti-LTF goat polyclonal antibody. Black arrows indicate strongly stained benign cells. In contrast, white arrows indicate negative staining of tumor cells (focal staining in less than 20% of CaP). In 30 of 30 (100%) cases, benign glands (black arrows) adjacent to cancer areas were highly positive for LTF. Magnification, 126. C, decreased LTF protein level in serum of CaP Patients. Serum was drawn from CaP patients (n = 34) undergoing their first diagnostic prostate biopsy, and from healthy males (n = 35). A significant two-fold decrease in mean LTF levels was observed in sera from CaP patients compared to controls ($p < 0.0001$) (ELISA). Whisker-box plot technique was used to illustrate serum LTF concentration by patient groups (CaP vs control). Students' t test confirmed a significant difference in LTF concentration between cancer and control groups (cancer vs control: 775.71 vs 1381.6, $p = 0.0001$).

performed. PI stained LNCaP cells were analyzed using an EPICS ELITE ESP (Beckman Coulter, Miami, FL) flow cytometer. Cell cycle analysis of DNA histogram is performed with ModFit LT software (Verity Software House, Topsham, ME). Experiments were carried out in triplicates.

RESULTS

LTF mRNA and protein levels are downregulated in prostate cancer. Evaluations of prostate epithelial cell (PEC) transcriptome using laser capture microdissection (LCM) derived benign and tumor epithelial cells and Affymetrix GeneChipÒ revealed LTF (downregulated in tumor cells) and AMACR (upregulated in tumor cells) as the most significant ($p < 10–6$) malignant cell-specific differential expression (Fig. 1A). CaP associated LTF expression alteration was validated at protein level. Significant downregulation of LTF protein was noted in all 30 whole-mount paraffin embedded formalin fixed tissue specimens of CaP patients analyzed by IHC (Fig. 1B). In these experiments an average of 20% focal tumor stains for LTF was detectable. Patients with organ-confined CaP (n = 34) had significantly lower levels of serum LTF ($p < 0.0001$) compared to control group with normal DRE and low PSA (n = 35) as determined by ELISA (Fig. 1C).

Quantitative assessment of LTF downregulation revealed an association with reduced PSA recurrence-free survival. LTF mRNA copy numbers normalized to GAPDH copy numbers were determined in 200 RNA specimens from laser micro-dissected matched tumor (T) and benign (B) prostate epithelial cells of 100 CaP patients. Decreased LTF expression was noted in tumor cells of 74.0% CaP patients, while 21% of CaP patients had increased LTF expression (Fig. 2A). As a quality control for the LCM-RNA specimens, a subset of RNAs from 20 benign and 20 tumor samples were analyzed for AMACR and GSTP1. As expected, AMACR overexpression (95%) and decreased GSTP1 expression (100%) was observed in virtually all tumor specimens (Supplementary Fig. 1). Low LTF "Tumor (T) / Benign (B)" expression (copy number) showed a significant correlation with shorter PSAR-free survival by Kaplan-Meier unadjusted analysis (p value ≤0.0322; Fig. 2B). Using chi-square analysis, a significant association